SOME OBSERVATIONS ON IMMUNE - ADHERENCE AND ITS ROLE IN PHAGOCYTOSIS OF VACCINIA VIRUS

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Some Observations on Immune-Adherence and Its Role in Phagocytosis of Vaccinia Virus

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INTRODUCTION

The response of the body to pathogenic microorganisms involves a complex interplay of cellular and humoral factors. The ingestion and destruction of infectious agents by specialized mesodermal cells of higher animals represents the end stage of a long evolutionary process.[1] The physicochemical aspects of phagocytosis are becoming increasingly well understood, and it is now evident that phagocytosis is dependent both on the environment in which the cell exists and on the nature of the foreign particle to which it is exposed.[1,2,3] *In vitro* and *in vivo* studies have shown the importance of various opsonizing factors in serum, and it is interesting to note that polysaccharides of virtually all infectious bacteria exert a positive chemotaxic action on the polymorphonuclear leukocyte.[1] While the precise sequence of events leading to destruction of pathogenic bacteria *in vivo* is still not clear, the primacy of the phagocytic mechanism is generally accepted. Wood and Smith feel that "man's ability to recover from acute bacterial infections depends in large measure upon the leukocytes that are formed in his bone marrow and accumulate in his blood".[4]

The role of the white blood cell and cells of the reticulo-endothelial system in viral infections is far less clear. As far back as 1909 von Prowozek and Yamamoto demonstrated elementary bodies of

vaccinia in stained leukocytes.[5] Whether this was infection or ingestion could of course not be determined. In the years following, many investigators attempted to explore the problem with results at marked variance with one another. Smith showed that vaccinia becomes associated with white blood cells of infected rabbits, but that these white cells even after washing will produce infection when injected into a second host.[6]

Fairbrother on the other hand showed a high degree of resistance to vaccinial meningoencephalitis by injecting white blood cells and immune serum simultaneously with the vaccinia. Both immune serum and either immune or normal white blood cells seemed essential; there was no protection when either immune serum or white blood cells was given without the other.[7] Jamuni and Holden demonstrated too that leukocytes aided in the inactivation of intracerebrally injected Herpes simplex in the presence of insufficient immune serum to neutralize alone.[8] The results presented by these workers seemed to suggest that in the presence of antibodies, the white blood cells played a contributory role in host resistance (phagocytosis?). Beard and Rous working with vaccinia and Kuppfer cells got results again suggesting the necessity of some humoral factor for phagocytic cells to demonstrate a protective effect.[9] While Kuppfer cells in vitro were readily infected with vaccinia, in vivo a combination of vaccinia and Kuppfer cells produced smaller lesions than were produced by vaccinia alone. Ginder using the fibroma virus showed an in vivo protective effect

only with leukocytes from immune animals. Mononuclear cells were more successful than polys, and he postulated that this might well be due to antibody precursors carried in the cells.[10]

Sabin felt that the protective effects of leukocyte-antibody combinations were merely the result of masking of an intracellular infection.[11] He demonstrated that leukocytes *in vitro* did indeed fix vaccinia, but that this vaccinia remained highly infectious for rabbits. Incubation of immune serum, leukocytes, and vaccinia *in vitro* decreased the infectivity of the virus, but this appeared to be due to adherence of immune serum to leukocytes, and washing of the white cells followed by ultracentrifugation rendered the precipitate again equally infectious. He demonstrated that *in vivo* even in immune animals, vaccinia could be demonstrated on washed leukocytes for up to twenty-four hours (the longest period used) after inoculation. This work was felt to indicate a purely passive role for animal leukocytes in viral infection and to demonstrate the greater importance of the neutralizing effect of anti-serum.

Florman and Enders extended the above work using tissue cultures of mononuclear cells which phagocytized indigo carmine particles.[12] These cells were readily infected by vaccinia, and anti-vaccinia-complement mixtures merely suppressed the infection. Immediately upon replacement of antibody-complement with non-immune serum, the infection reappeared. Each of their experiments was carried out for at least three weeks. These authors proposed that the results of Fairbrother[7] and Jamuni and Holden[8] were due to the short duration of

their experiments.

Merling utilized a different approach examining white blood cells scraped from the cornea of a rabbit eye infected in vivo with vaccinia.[13] He was able to show a sequence of events remarkably similar to that described for classic bacterial phagocytosis with chemotaxis, adherence of the vaccinia to the cell surface, and ingestion.[1] While he felt that this was truly phagocytosis, when these cells disintegrated, vaccinia particles were released which continued to grow for three weeks. One wonders in light of the physical similarity between phagocytosis and virus penetration and subsequent infection[14] whether Merling might not have been observing an infection of white blood cells. This work and that of Florman and Enders[12] suggests the conclusion of the latter that during virus infection white blood cells might serve as a repository for latent infection temporarily masked by antibody.

Smorodintsev tried to distinguish between host resistance to bacterial and viral infections.[15] He attempted to induce phagocytosis of tick-borne encephalitis virus by giving a priming intraperitoneal injection of 10% peptone broth in mice causing a white cell exudate. These animals subsequently showed no greater resistance to intra-peritoneally injected virus than did normal controls. He extended the work by introducing chick erythrocytes with adsorbed influenza virus intraperitoneally. Despite rapid phagocytosis of the red cells, there was no decrease in magnitude of infection. He ascribed host resistance to specific neutralizing antibodies which caused virus to lose the ability to attach to cells; this free virus could then be destroyed by the body

temperature which he showed to be unfavorable for survival. While he presents little data in his paper, that evidence which he does give on the failure of phagocytes to alter the course of infection is most suggestive.

Boand, et al. working with the much smaller virus of influenza (85 mμ) have presented the most critical data to date for the occurrence of phagocytosis in vitro and in vivo.[16,17,18] They incubated influenza with white blood cells and serum from either control or hyperimmune rabbits. They then stained the preparations with fluorescein labeled anti-rabbit globulin and observed the localization of fluorescence within white blood cells. Those preparations utilizing immune serum showed marked fluorescence associated with the leukocytes which they felt for three reasons was phagocytosis:

1) Fluorescent particles were irregularly clumped in the white blood cells.
2) Ultra-centrifugation of lysed cells showed fluorescence to be associated with the cytoplasm.
3) Inhibition of phagocytosis by low temperature or ethanol inhibited the reaction.[16,17]

In vivo studies using the fluorescent antibody technique showed influenza injected intraperitoneally to be more rapidly removed from peritoneal fluid in immune animals and to be associated with leukocytes.[18] The questions their work leaves unanswered is the fate of ingested virus (it is rendered non-infectious), and the role of antibody in their system. As Smorodintsev[15] suggests, the commonly ascribed role of

antibody in viral infections is to prevent adsorbtion to the host cell. Yet these workers have demonstrated both antibody and virus within the host cell.

The importance of opsonizing factors in enhancement of bacterial phagocytosis has been shown.[2] Wood points out that encapsulated bacteria generally resist phagocytosis *in vitro* unless previously sensitized with specific antibody.[20]

A complex immunological system also has been described by Nelson--immune adherence--which seems markedly to increase phagocytosis of many different micro-organisms beyond the action of antibody and complement alone.[21,22,23] As Nelson now defines the phenomenon, it involves the *in vitro* reaction of a variety of bacteria, [21] viruses,[24,25] and soluble antigens[26] sensitized by homologous antibody and complement with primate red blood cells causing an attachment of this antigen complex to the erythrocyte surface. As a demonstration of this enhancement of phagocytosis, *T. pallidum* (sensitized with antibody)→defibrinated blood which contains complement → clear supernate with 20% of the original spirochetes adherent to erythrocytes, while *T. pallidum* (sensitized with antibody)→complement)→washed human erythrocytes → 97% of the original spirochetes adherent to erythrocytes and a clear supernate.[21] The difference was felt to represent phagocytosis of *T. pallidum* by white blood cells. Of course it could also be clumping on white cell surfaces or simply a sedimentation effect.

Immune-adherence with primate erythrocytes was probably first

described by Duke and Wallace in 1930.[30] These workers were attempting to find a test for individuals with occult trypanosomiasis. They found that one drop of a suspension of trypanosomes mixed with one drop of blood from an infected individual resulted in agglutination of red blood cells. The necessary factor in the blood of infected individuals which was most probably antibody, they called "adhesin." The reaction of immune-adherence is felt by Lamanna to be one of several indicator reactions requiring antigen, antibody, and complement--the prototype being the Rieckenberg phenomenon involving guinea pig platelets, antibody, complement, and V. cholerae with adhesion occurring.[28] While Nelson and Nelson claim that, with the exception of guinea pig platelets and primate red blood cells, adherence is a result of failure to absorb antibody to the indicator particle, experiments have not been sufficiently well controlled either to refute or substantiate this thesis.[29]

Because of the considerable uncertainty still surrounding the possibility of phagocytosis of a large virus, it was decided to attempt to re-explore the problem utilizing vaccinia with the immune-adherence system which appears to offer optimal conditions for virus ingestion.

Vaccinia seemed for many reasons to be an appropriate virus to select. Its relatively large size (260 mμ)[19] makes possible direct visualization with dark-field microscopy, and the bulk of previous work on phagocytosis had employed the virus. Equally important, it is an animal pathogen, and it was hoped that it might be possible to distinguish between infection of the white blood cell and actual phago-

cytosis as well as to draw conclusions as to implications in human resistance to vaccinia. It was felt that the fate of the virus could be determined in a way analogous to that of Boand, et al. using fluorescent antibody.[16]

As a corollary to the above plan, it was felt that additional information regarding the properties and range of use of the immunologic system involving immune-adherence with particular reference to viruses would become available.

MATERIALS AND METHODS

Virus: The vaccinia used was a testicular strain originally obtained from the laboratory of Francisco Duran-Reynals. This had been grown on a rabbit's back and when its purification was begun, it was said to have had an ID_{100} for rabbits at a dilution of 1/625.

Since anti-serum was to be prepared from rabbits, it was felt advisable to prepare vaccinia that had been passed through another host to avoid the possibility of homologous antibodies interfering with the immunologic reaction. Consequently virus was utilized following four or five passages on the chorioallantoic membrane (CAM) of eggs using a modification of the technique described by Overmann and Tamm.[32] This involved suspending CAM's in a 10% solution of isotonic saline buffered with phosphate to give a pH of 7.4. The solution was homogenized with a Potter-Elvingham homogenizer under sterile conditions and then centrifuged at 2000 rpm for 15 minutes in

a refrigerated centrifuge at 4°C. The supernate was pipetted off and ten-fold dilutions were made in phosphate-buffered saline (PBS). Chorioallantoic membranes of eleven day sex-linked eggs supplied by Hall Brothers Farms were inoculated by syringe with either 0.25 ml or later 0.2 ml of each dilution--generally five eggs per dilution. The eggs were incubated at 37°C for three days after which they were opened and pocks counted. The titer was determined from those dilutions giving consistent results with from 5-50 pocks per CAM. Vaccinia for additional passage was prepared as above from chorioallantoic membranes showing confluent growth. Initially the membranes were stored in phosphate-buffered saline with penicillin 50 units per ml and streptomycin 50 µg/ml at -14°C. The antibiotics were later found not to be necessary at this temperature. There was found to be little variation in number of pocks at a given dilution, and there was an average death of around 10% of the eggs.[32] The initial titer of the vaccinia before egg passage was 8×10^5 pock-forming units (pfu)/ml and after a series of four passages, the titer of that vaccinia to be purified was 1.6×10^7 pfu/ml.

Since Nelson and Nelson have presented evidence that immunoadherence is sufficiently sensitive to be altered by small quantities of extraneous substances reacting as either antigen or antibody in a reaction mixture (mixed aggregation),[29] it was felt necessary to have preparations of vaccinia as pure as possible. Initially a modification of the differential centrifugation technique first described by Craigie

was used.[33] This involved the following procedure:

50 ml of vaccinia in phosphate-buffered saline (concentration of 3×10^6 pfu/ml) were spun for 10 minutes at 2000 rpm at 4°C. The supernatant fluid was then spun at the same speed for 15 minutes. This supernatant fluid was spun for 15 minutes at 16,000 rpm at 4°C in a Servall high-speed centrifuge. The precipitate was resuspended in 50 ml of medium and spun for 10 minutes at 2000 rpm. The alternation of high- and low-speed centrifugation was repeated three times more and the precipitate resuspended in a final volume of 25 ml phosphate-buffered saline giving a suspension with an expected titer of 6×10^6 pfu/ml. Marked difficulty was noted throughout in resuspending the precipitate from the high-speed centrifugation. By CAM titration the concentration of virus was found to be only 6.5×10^3 pfu/ml. Consequently it was decided to use a fluorocarbon purification first described by Gessler, et al.[34] Homogenizing virus and contaminants at high speed with the fluorocarbon Genetron (trifluorotrichloro-ethane) was shown to result in removal of the non-viral protein from the aqueous layer. The method that was utilized was that of Epstein with minor modifications.[35] Weighed chorioallantoic membranes were diluted 1:2.5 with sterile phosphate-buffered saline and Genetron was added in a ratio of 1:2 of buffer. The resultant mixture was homogenized in an ice bath using an Omni-Mixer at a scale setting of 75 for sixty seconds. The homogenized suspension was centrifuged for three minutes at 1500 rpm (approximately 300g) at 4°C. The aqueous supernate was put aside

and half the initial volume of both buffer and Genetron was added to the residue. This mixture was homogenized and centrifuged as above. The two aqueous layers were combined, a quantity of Genetron equal to that added in the second step was added, and the homogenization and centrifugation were again repeated. This cycle was repeated twice, and the final aqueous layer was used as purified virus. The preparation appeared dense and regular under dark-field microscopy as contrasted with diluent alone. The purified preparations that were titrated showed little loss of activity as compared to their initial titers on chorioallantoic membranes and ran approximately 1.25×10^7 pfu/ml. Purified vaccinia was quick-frozen and stored in a carbon dioxide chest at $-56^{\circ}C$.

Antibody: Antibody was produced by immunizing three rabbits with a testicular strain of vaccinia virus. 20 ml of blood for normal serum was drawn by cardiac puncture from each. Then 2 ml of virus ($ID_{100}=625$) were scratched on the back of each rabbit; four days later 0.25 ml of a 1/75 dilution of virus was given intradermally; this was followed in 3 days by 3 ml of virus intravenously, then 4 days later another 3 ml intravenously, and in 3 days 30 ml were removed by cardiac puncture completing the first course. The second course, which began two days after the first course ended, consisted of three injections of 3 ml of virus at a 1/25 dilution given every third day--the first intraperitoneally, the second two intravenously. One week after the last injection, the animals were bled out by cardiac puncture.

All anti-serum was centrifuged at 10,000 rpm for one hour at

0°C and fat particles were removed. The antiserum was also heated to 56°C for thirty minutes to inactivate complement, and then stored at -4°C.

Complement Fixation: The technique of complement fixation was a modification of that described by Kabat and Mayer.[36] Sensitized sheep red cells (EA) were prepared by adding an equal volume of hemolysin at 1/500 dilution previously standardized by R.A. Nelson, Jr., to 5% sheep erythrocytes washed three times in serum albumin veronal buffer with Ca^{++} and Mg^{++} ($SAVB^{++}$)[37] and allowing the mixture to stand for thirty minutes at room temperature. Guinea pig complement (Carworth Farms) was titrated, and it was found that 0.5 ml of 1/72 dilution contained 50 H_{50} units (the dilution subsequently used). 0.2 ml of antiserum at various dilutions was added to 0.5 ml of a 1/72 dilution of complement and 0.5 ml of vaccinia at specified dilutions. This reaction mixture was either incubated at 37°C for 45 minutes or at 4°C for sixteen hours with comparable results. Then 0.3 ml of sensitized sheep cells (EA) was added, incubated for 45 minutes, and degree of hemolysis graded 1+ to 4+ was read visually. The dilution of antibody required to decrease hemolysis to 50% in the presence of excess antigen was determined using hemolysin standards as controls.

Immune-Adherence:

Complement: Pooled guinea pig serum (Carworth Farms) was used as the complement source in all experiments. Since Nelson and Nelson felt that heterologous antibodies to human erythrocytes present in

guinea pig serum might interfere with the reaction,[29] all serum was absorbed with human red blood cells by incubating 10 ml of undiluted serum with 0.5 ml of packed human type O erythrocytes at 0°C for fifteen minutes, then removing the cells by centrifuging for twenty minutes at 4°C at 16,000 rpm. The absorbtion was repeated twice.

Complement was titrated in a standard immune-adherence system consisting of a soluble antigen of S. typhosa at a dilution of 1:1000 and antiserum at 1:100. This had previously been shown by Nelson to supply both antigen and antibody excess and to make the reaction dependent upon complement. A 1.5% suspension of human red blood cells was used. A reaction mixture of 0.2 ml antigen, 0.2 ml antibody and 0.2 ml of complement at various dilutions was incubated at 37°C for ten minutes; then 0.1 ml of the red blood cell suspension was added, the solution mixed by shaking and incubated for 45 minutes at 37°C at which time patterns were read visually as described by Nelson and Woodworth.[24] 0.2 ml of complement at 1/68 absorbed with red blood cells gave a 2+ reading implying 3400 IA_{50} units per ml compared with 3750 IA_{50} units in unabsorbed complement. The values were similar to those obtained by other workers.[37]

In the standard protocol used for immune-adherence, 0.2 ml of complement at a 1:36 dilution was used supplying 2-30 IA_{50} units per reaction mixture. This is in the range recommended by Nelson and Nelson.[37]

Antigen and Antibody: These were prepared as previously described.

Diluent: The diluent used was $SAVB^{++}$ prepared as described by Nelson and Nelson.[37]

Erythrocytes: Human type O blood was collected from a donor and stored in modified Alsever's solution[37] at 4°C for a maximum of four weeks. As needed, erythrocytes were washed three times, once in isotonic saline and twice in $SAVB^{++}$, each time removing the buffy coat following centrifugation, and standardized so that 1 ml of erythrocytes in 10 ml of water gave an optical density of 0.395 at a wavelength of 0.541 mu on a Beckman spectrophotometer when a 2% solution was desired or an O.D. of 0.300 at 541 mu for a 1.5% solution following a previously established standardization curve.

Reaction Mixture: The method for producing immune-adherence was as follows: 0.5 ml of antibody dilution was added to 0.2 ml antigen dilution drop by drop at room temperature. After five minutes' incubation, 0.2 ml complement at 1:36 dilution was added, the tubes shaken and incubated for ten minutes at 37°C. Then 0.1 ml of a 1.5% suspension of human erythrocytes was added to each tube and the tubes were incubated for 45 minutes for S. typhosa and approximately 60 minutes for vaccinia at 37°C with shaking for only the first five minutes.

All glassware was acid-cleaned with concentrated sulfuric or nitric acid.

Ouchterlony Agar Diffusion Plates: The Ouchterlony method of agar diffusion was used in an attempt to gauge the degree of purification achieved by fluorocarbons. Plates were prepared in a modification of

the technique described by Wilson and Pringle.[38] 15 ml of a 0.75% solution of Noble agar in veronal buffer with Mg^{++} and Ca^{++} containing 0.1% merthiolate and 0.2% methyl orange were poured per petri plate, and antigen was added to outer cups drilled with a cork bore in a radius 1.5 cm from a center cup filled with either antigen or commercially prepared egg albumin. The plates were kept at 4°C until precipitin lines appeared--a matter of two weeks.

Fluorescent Antibody: It was hoped that it would be possible to detect vaccinia within white blood cells by a modification of the fluorescent antibody technique described by Coons and Kaplan.[39] Some preliminary work was done utilizing immune-adherence mixtures showing optimal adherence visually. To 1 ml of this reaction mixture was added 1 ml of a suspension of white blood cells harvested from the guinea pig peritoneal cavity. These cells were from an exudate induced by injecting 30-50 ml of sterile saline intraperitoneally eight hours before removal, then giving an additional 30 ml priming dose one hour prior to harvesting, and then harvesting the cells in a special tube with a capillary bore and large bulb. The immune-adherence-white blood cell preparation was incubated in a slow rotator (6-10 rpm) at 37°C for thirty minutes, and then samples were smeared on microscope slides. These slides were fixed in absolute ethanol or acetone for fifteen minutes at room temperature or dried directly in air. One drop of commercial anti-rabbit globulin (Sylvana) diluted 1:10 was added to the fixed preparation; and the slide was stained at room temperature in a cotton-moistened Petri plate for thrity minutes, washed in phos-

phate-buffered saline three times over a period of ten minutes, moistened with one drop of buffered glycerol, covered with a cover slip, and observed under a fluorescent microscope.

RESULTS

Immune-Adherence: Immune-adherence assays were run with vaccinia five times in the course of the work. As will be discussed later, the last two assays were probably carried out with insufficient antigen and the titrations produced no visible or microscopic immune-adherence.

Assay 1: The reaction mixture of this assay alone was varied from that described under materials and methods. It consisted of:

virus (stock 1)	0.2 ml
antibody	0.2 ml
complement (1/20)	0.5 ml
erythrocytes 2%	0.1 ml

Other steps of the procedure were carried out in a standard fashion. The results are presented in table 1. It was felt at the time that the patterns were extremely hard to read and while the controls were negative, suggesting that the reaction was a specific one, any quantitative interpretation of the results is probably not warrented.

Assay 2: In the following two assays the reaction mixture was as described in the protocol:

virus (stock 1)	0.2 ml
antibody	0.5 ml
complement (1/30)	0.2 ml
rbc's 1.5%	0.1 ml

Taverne assumed that visual hemagglutination could only be obtained when the ratio of antigen particles to red blood cells approached 1:1.[25] It did seem reasonable that a decrease in erythrocytes would make settling slower and patterns easier to read. For this reason 1.5% erythrocytes were used in the remaining work. The second assay was perfectly satisfactory from a technical standpoint; the patterns were easily read. While quantitative counts were not done under the dark-field microscope, adherence was seen and appeared well correlated with the visual observations. There was no adherence microscopically in any of the controls.

Assay 3: The third immune-adherence reaction was again technically satisfactory and demonstrated that all components must be present in sufficient quantities for the phenomenon to occur. The effect of antibody excess can be seen in the columns with high concentrations of antibody in table 3. Unfortunately the vaccinia preparation was a fresh purification which was never adequately titered on chorioallantoic membranes (vaccinia stock 2). It seems reasonable to assume, since antigen concentration is the only variable from assay 2, that the presence of antibody excess here and not in the second assay implies a lower concentration of vaccinia in this assay.

A Comparison of Titer of Antibody Using Complement Fixation and Immune Adherence: Antiserum was titrated as described previously by the complement fixation technique. Tables 4 and 5 show the results of two titrations; the first over a large range and the second with

IMMUNE-ADHERENCE CHECKERBOARDS

TABLE 1

vaccinia(stock 1)		antibody--dilutions of immune rabbit serum							
dil.	pfu present in reaction mixture	1:4	1:16	1:64	1:256	1:1024	1:4096	1:16384	Buff.
und.	2.5×10^6	4	4	4	4	--	2	1	0
1:5	5×10^5	4	4	4	3	2	2	0	0
1:10	2.5×10^5	4	4	4	3	0	0	0	0
1:20	1.3×10^5	4	4	4	2	2	2	1	0
1:40	6.3×10^4	0	0	2	2	0	2	3	0
1:80	3.2×10^4	0	0	0	0	0	0	0	0
Buffer		0	0	0	0	0	0	0	0

TABLE 2

(stock 1)		1:4	1:16	1:64	1:256	1:1024	1:4096	1:16384	Buff.
und.	2.5×10^6	3.5	4	4	T	0	0	0	0
1:5	5×10^5	3.5	4	3	0	0	0	0	0
1:10	2.5×10^5	3	3.5	3	T	0	0	0	0
1:20	1.3×10^5	0	0	0	0	0	0	0	0
1:40	6.3×10^4	0	0	0	0	0	0	0	0
1:80	3.2×10^4	0	0	0	0	0	0	0	0
1:160	1.6×10^4	0	0	0	0	0	0	0	0
Buffer		0	0	0	0	0	0	0	0

TABLE 3

vaccinia(stock 2)

dil.	1:4	1:16	1:64	1:256	1:1024	1:4096	1:16384	Buffer
und.	T	0	T	1	1	1.5	0	0
1:2	0	0	T	3	T	0	0	0
1:4	0	0	2.5	3.5	0	0	0	0
1:8	2	3	4	3	1	0	0	0
1:16	2.5	3.5	4	4	2	T	0	0
1:32	1	2	3.5	4	1.5	T	0	0
1:64	0	1	2.5	2	0	0	0	0
1:128	T	T	2.5	1	0	0	0	0
Buffer	0	0	0	0	0	0	0	0

COMPLEMENT FIXATION

(EXPRESSED AS DEGREE OF HEMOLYSIS FROM 1+ to 4+)

TABLE 4

impure vaccinia dil.	pfu present in reaction mixture	antiserum (0.2 ml at each dilution) 1:5	1:25	1:125	1:625	1:3125	Buffer
1:5	$5x10^6$	0	0	2	4	4	4
1:25	10^6	0	0	3-4	4	4	4
1:625	$2x10^5$	0	1	4	4	4	4
1:3125	$4x10^4$	3	3	4	4	4	4
Buffer		4	4	4	4	4	4

TABLE 5

		1:10	1:20	1:40	1:80	1:160	1:320	1:640	Buffer
1:10	$2.5x10^6$	0	0	0	0	T	2	3	4
1:20	$1.25x10^6$	0	0	0	0	0	2	3	4
1:40	$6.2x10^5$	0	0	T	T	1	2.5	3.5	4
1:80	$3.1x10^5$	0	0	0	T	1	3	4	4
1:160	$1.5x10^5$	0	0	0	T	2	3.5	4	4
1:320	$7.5x10^4$	0	0	0	T	2	4	4	4
1:640	$3.8x10^4$	0	0	0	1	3	4	4	4
Buffer		4	4	4	4	4	4	4	4

NATURAL ANTIBODY IN GUINEA PIG COMPLEMENT AGAINST VACCINIA IN IMMUNE-ADHERENCE

TABLE 6

Purified Vaccinia		Complement				
		1:10	1:20	1:40	1:80	1:160
1:5	5×10^5	0	0	0	0	0
1:	1.3×10^5	0	0	0	0	0
1:80	3.2×10^4	0	0	0	0	0
1:160	1.6×10^4	0	0	0	0	0

much closer dilutions designed to establish the $C'H_{50}$ value (that quantity of antiserum decreasing hemolysis to 50% in the presence of excess antigen and complement) for the antiserum. The $C'H_{50}$ for the antiserum is readily calculated from table 5 to be 1:1600 per ml. In the complement fixation assay homogenized, but not purified vaccinia of known titer was used. An inspection of table 3 shows that the comparable $C'IA_{50}$ (50% of maximal agglutination) for the antiserum is 1:5110.

Ouchterlony Plates: The agar-diffusion technique was utilized in an effort to find out the extent to which fluorocarbon purification removed the foreign protein present in the vaccinia (egg albumin). The reaction is a classic precipitin reaction carried out in an agar medium and possibly allowing the detection of different antigen-antibody systems. As is shown in Plate I, antigen-antibody precipitation occurred only with the egg albumin-anti-egg albumin system and to a lesser extent with the impure vaccinia-egg albumin system. Since the titers of impure and pure vaccinia were of the same order, it can at least be said that sufficient egg albumin was removed so that a precipitin reaction no longer was demonstrable.

It was hoped that if there were changes in antigenicity of vaccinia resulting from purification, these might also be demonstrated by agar diffusion. No precipitation was seen in either the pure or impure system run against undilute antibody to vaccinia.

Fluorescent Antibodies and Phagocytosis: One attempt was made to show phagocytosis by using fluorescent antibody in an immune-adherence

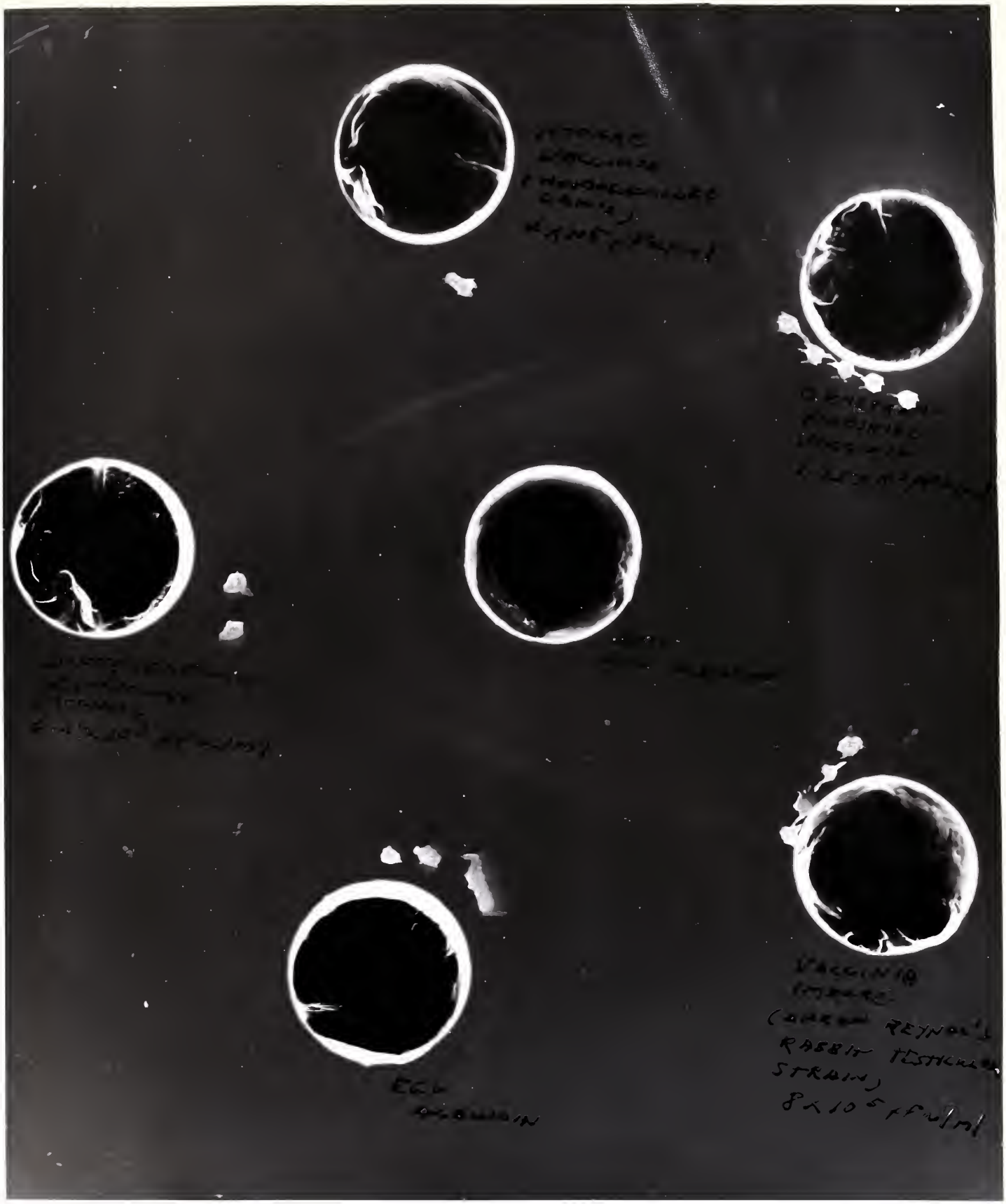

PLATE I

system operating adequately. The procedure was as outlined under Materials and Methods. The immune-adherence mixture chosen was one giving a 4+ reaction in the third assay (vaccinia 1:16, (Stock 2) antibody 1:64--see table 3). This reaction mixture showed maximal hemagglutination in four identical tubes during the course of the fluorescent antibody experiment while controls without either antigen, antibody, or complement demonstrated no hemagglutination. The white cell count of the harvest from the guinea pig peritoneum was $3600/mm^3$ with 54% polymorphonuclear leukocytes. This meant that there were 3.6×10^6 white blood cells and approximately 10^5 vaccinia pock-forming units in the reaction mixture. The titer of vaccinia was not known and this can only be an estimate based on the results of the first two assays. Slides were observed under the fluorescent microscope on the day of preparation and on each of the two days following. At no time was any fluorescence seen associated with leukocytes in either the complete system or in its control.

Demonstration of Absence of Anti-Vaccinia: Other work in this laboratory showed appreciable antibody to bacteriophage present in guinea pig serum (complement) at a dilution as great as 1:80. Taverne had felt this to be antibody to E. coli contaminants in phage preparations.[25] Assays were carried out to determine whether natural antibody to vaccinia could also be demonstrated in guinea pig serum by immune-adherence. Each reaction mixture contained:

vaccinia dilution	0.2 ml
g.pig serum (as Ab and C^1)	0.2 ml
human erythrocytes 1.5%	0.1 ml
$SAVB^{++}$	0.5 ml

The results are shown in table 6. This demonstrates quite clearly the absence of antibody in guinea pig serum reacting in immune-adherence with vaccinia virus.

DISCUSSION

The results indicate that the immune-adherence technique is applicable to the vaccinia virus if sufficient antigenic particles (here equated indirectly with infectious units) are present. In this work a requisite number of pock-forming units seemed to lie in the region of 5×10^5 per assay tube--a ratio of one pfu/300 red blood cells. Since relative quantities of reactants were varied in the only two assays where absolute numbers of virus particles were known, no comment can be made regarding the reproducibility of the technique.

The inability to demonstrate immune-adherence in the last two assays requires explanation. Following the completion of the third assay, the reagents were stored as outlined in Materials and Methods for over five months. The fluorocarbon-purified vaccinia (stock 2) was stored in phosphate-buffered saline in sealed glass ampules at $-56^{0}C$ in a carbon dioxide chest without added protein. Despite the fact that no visual clumping was evident, it proved impossible upon rethawing the vaccinia either to grow it on chorioallantoic membranes or to cause it to react in immune-adherence. Since the complement

retitered at the same value as before, human erythrocytes remained reactive in bacteriophage and typhosa immune-adherence systems, and rabbit serum is known to be extremely stabile as it was stored; it is certain that the vaccinia had lost both its infectivity and antigenicity. It is generally known that most animal viruses, including vaccinia lose infectivity when stored under these conditions without protein. Other work with fluorocarbon purified vaccinia has shown that another factor which decreases infectivity is clumping of the virus (McCrea, J., personal communication). Collier has reviewed the difficulties of storing vaccinia in any but dried form and the highly deleterious effect of an acid environment, which could well have occurred with poor sealing of the glass ampules in the carbon dioxide chest, is especially noted.[40]

Dubos indicates that 0.12-0.4 µg antibody nitrogen is required for measurement of a 50% hemolysis endpoint in complement fixation with pneumococci, whereas a similar immune-adherence endpoint for pneumococci requires only 0.015 µg antibody nitrogen--roughly one-tenth as much antibody nitrogen.[41] Nelson stresses this heightened sensitivity as one of the advantages of the immune-adherence phenomenon for immunologic work.

The comparative results with vaccinia show that in complement fixation, the $C'H_{50}$ for the antiserum occurs at a dilution of 1:1600 of antiserum, while in immune-adherence a $C'IA_{50}$ is found at a 1:5110 dilution of antiserum. Since the proportions of antigen, antibody,

and complement were different in the two assays, and no quantitative antigen nitrogen determinations were done, comparison is difficult. It is evident though that the immune-adherence assay detected a quantity of antibody one-third that demonstrable by complement fixation, suggesting the greater sensitivity of immune-adherence as compared with complement fixation in the vaccinia system.

The particular virtue of gel diffusion techniques over the classic tube precipitin reaction is the possibility of separation of reaction zones in a system containing multiple antigen-antibody complexes. This stems from two properties inherent in immunologic systems. First, rate of diffusion of either antigen or antibody is dependent upon the size of the molecule relative to the gel matrix and the electrical charge of the molecule. Secondly, precipitation is confined to a zone where antigen and antibody are present in the optimal proportions necessary for visible precipitation.

It had been hoped that the Ouchterlony technique would make possible some inferences concerning the effect of Genetron on the antigenicity of vaccinia. Unfortunately, no visible precipitation occurred in any system involving vaccinia-anti-vaccinia. Since antibody is freely diffusible and was used undiluted, it is likely that the failure to show a precipitin reaction resulted either from the presence of too few antigenic particles, or the use of too dense an agar matrix. It would be interesting to repeat the work using Ion Agar which can be used at one-half the concentration of Noble agar.

The technique did indicate removal of egg albumin impurities from the vaccinia preparation in the course of the purification.

The work of Nelson and others has clearly demonstrated immune-adherence to be a highly sensitive and reproducible method for measuring antigen or antibody in a given system. The present work demonstrates the greater sensitivity of this reaction as compared to complement fixation and precipitin reactions, suggesting clinical applications in detection of occult disease where more commonly used immunologic techniques are not successful. Since multiple antigen-antibody systems are present per vaccinia particle, the relationships of the antigen-antibody complexes operable in this system to those functioning in other immunologic tests still remain to be determined. Difficulties inherent in the procedure are demonstrated in this work. The relatively high concentration of purified antigen required presented the most significant problem. Nelson,[21] Taverne,[25] and the present work all showed that for microorganisms of considerably different size, 10^6-10^7 particles were necessary, with identical erythrocyte concentrations, for immune-adherence. Visual hemagglutination hence seems to require a constant number of bonds per system, and it would appear that more bonds per pair of erythrocytes are not necessary with smaller particles for visualization of the reaction. Until work has clarified the distinction raised by Nelson and Nelson between mixed aggregation due to antibodies to impurities in the system and immune-adherence,[29] it will be necessary to remove all foreign protein from the antigen solution.

The present work gave no answer to the original question of whether phagocytosis of vaccinia virus occurs. The technical aspects of fluorescent antibody layering in this system remain to be worked out. Even with the staining technique operable, there still remains the possibility that phagocytosis of a larger virus differs from that of influenza[17] and involves only the ingestion of the virus without antibody. If this were the case, an antibody prepared to the original antibody would give no indication of the intracellular location of the virus. A better approach might be to use the more direct technique of fluorescently staining the DNA of vaccinia with acridine orange.[31] Since the acridine orange fluorescence is of a different color for DNA than RNA, if virus were present in the cytoplasm, it should stand out clearly against the RNA background.

The most important aspect in studying the relationship of virus to white blood cells is the ultimate fate of the virus. The work reviewed in the introduction seems to follow a pattern implying that in the presence of antibody there occurs an intimate association between the phagocytic cell and the virus. The question not explored thoroughly by any workers was whether this association--phagocytic or infectious--was to the benefit or detriment of the host. Even if phagocytosis occurs, the situation might be as it is with certain chronic bacterial infections (e.g. tuberculosis, brucellosis, and typhoid fever)[20] where the pathogen is not destroyed, but acts as an intracellular parasite. Then the association between leukocyte and virus might serve as both a

method of transport and protection for the invader. Clinically a transient viremia occurs 6-8 days after vaccination, but neutralizing antibodies do not develop until 12-14 days after inoculation. Individuals with adequate gamma-globulin never get generalized infections.[42] It is interesting to speculate as to whether the virus might be in leukocytes in the days between the viremia and appearance of neutralizing antibodies. Therefore if vaccinia can be demonstrated in guinea pig leukocytes, it would then be necessary to grow these cells under optimal conditions for survival and study the infectivity within the cell upon its death.

SUMMARY

Immune-adherence of vaccinia virus was demonstrated using Genetron purified viral preparations. The previously reported increased sensitivity of the immune-adherence assay as compared to the complement fixation assay was shown to apply to vaccinia. By the use of the Ouchterlony agar diffusion technique, it was demonstrated that Genetron purification of vaccinia removes egg albumin from the impure preparation. It was impossible to show changes in antigenicity of the virus occurring with purification, and reasons for the lack of success were discussed. Absence of antibody in guinea pig serum reacting in immune-adherence with vaccinia virus was demonstrated. A method for fluorescent antibody staining of anti-vaccinia antibody was described; its applicability to demonstration of vaccinia phagocytosis and reasons for failure were discussed as were other approaches to experimental proof of viral phagocytosis *in vitro* and the applicability to human infection.

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